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FINE STRUCTURE AND COMPOSITION OF A SILICEOUS SPONGE SPICULE[1]

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When a siliceous skeletal spicule of a marine sponge is dissolved in hydrofluoric acid (HF), a filament remains. We report here a new understanding of the structure and composition of the siliceous spicule and its axial filament. Bütschli (1901) showed that these filaments stain with several organic dyes and behave toward a variety of reagents as protein. He concluded that the HF-resistant residue, presumably all axial filament, contained a very small amount of organic material around which silica deposition is initiated. The view that the *whole* axial filament, or even a major fraction of it, is "sans doute protéinique" (page 497 Lévi, 1963) appears to be a misreading of this statement by Bütschli. Bütschli further showed that spicules fractured in cross section and stained with various dyes exhibit a triangular core and several regularly spaced concentric rings. The laminar structure has been widely documented for large spicules. The early literature is exhaustively reviewed by Minchin (1909).

Drum (1968) applied rotary replication techniques in the study of the surface ultrastructure of siliceous spicules. In his electron micrographs he points out a filament that remains after HF removal of the spicule. From its binding of dyes and its loss on ultramicroincineration he concluded that the filament was an organic axial filament, primarily carbohydrate. He did not obtain enough material for direct chemical analysis.

We present three kinds of data on the structure and composition of the axial filament and the surrounding siliceous spicule. These are direct chemical analysis, phase-contrast light micrographs, and electron micrographs obtained by both direct (rotary shadow) and indirect (negative replica) procedures. Our major conclusion is that there is sufficient carbon in the siliceous spicule to provide a major fraction of the visible axial filament as a protein or carbohydrate. Nevertheless, organic matter is only a small fraction of the HF-resistant residue. This conclusion is not contrary to that of Bütschli, but is contrary to that of more recent workers who have cited Bütschli as their authority. The analytical methods used here were not available to Bütschli and have not been combined in this manner by others.

Materials and Methods

Growth and maintenance of sponges

Clumps of marine sponge were obtained through Pacific Biomarine from the coast of California near Los Angeles. These were held with sea urchins

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and other marine animals in a recirculating aquarium (Instant Ocean) at 13° in artificial sea water (Instant Ocean) to which has been added sodium silicate to give 1 m*M* silicate. Growth of new tissue occurred in this aquarium. The sponge was identified as to species on the basis of morphology and spicule type. *Acarnus erithacus* has a peculiar acanthocladotyle as a minor macrosclere (DeLaubenfels, 1932). The spicules studied were the major macrosclere, a simple style.

Preparation of spicules

Spicules were freed from the surrounding sponge tissue and other contaminants in several ways: (a) nitric acid and density gradient centrifugation, (b) nitric acid and water washing, (c) crude enzymatic digestion and water washing. For light microscopy and some of the chemical analysis, spicules were freed from the sponge tissue by digestion in boiling concentrated nitric acid. The acid was washed off by repeated centrifugation through distilled water. The spicules were separated from organic debris and sand by isopycnic centrifugation on a density gradient of carbon tetrachloride and ethylene dibromide. They were washed in methanol and dried at 20°. For electron microscopy spicules were cleaned in concentrated nitric acid at room temperature (4 hours) and washed in distilled water. Finally the styles were separated from the other spicules by differential settling in distilled water and air dried at 60°.

Chemical analysis

Acid-washed, density gradient spicules were freed from tiny flakes (presumably mica) of the same density (1.93 to 1.96) by gentle swirling in a shallow dish. The spicules were dried from methanol in a tared Teflon weigh boat. The spicule cake was flooded with 1 *N* HF and held at 55° on a water bath to dryness. This was repeated to constant weight of the residue at 55°. Portions of the weighed, air-dried residue were subjected to three different analyses: (a) gas chromatography for C, H, and N, (b) emission spectrography, and (c) atomic absorption.

Light microscopy

Nitric acid-washed spicules were observed by phase-contrast light microscopy in a Wild M20 research microscope. The photographs in this paper were made with a 100 × oil immersion objective on Kodak Panatomic-X 35 mm film. Spicules were placed between a plastic cover glass and a plastic dish and individual spicules observed from the time the HF reached them. Timed observations thus represent time after contact of the observed spicule with the etching fluid. A spicule and its remnants were observed for as long as 2 hours.

Electron microscopy

Drum (1968) developed a procedure using rotary shadow to make a replica from which the spicule was then removed by HF digestion. This three-dimensional replica allowed him to study the surface well, but the internal filament was

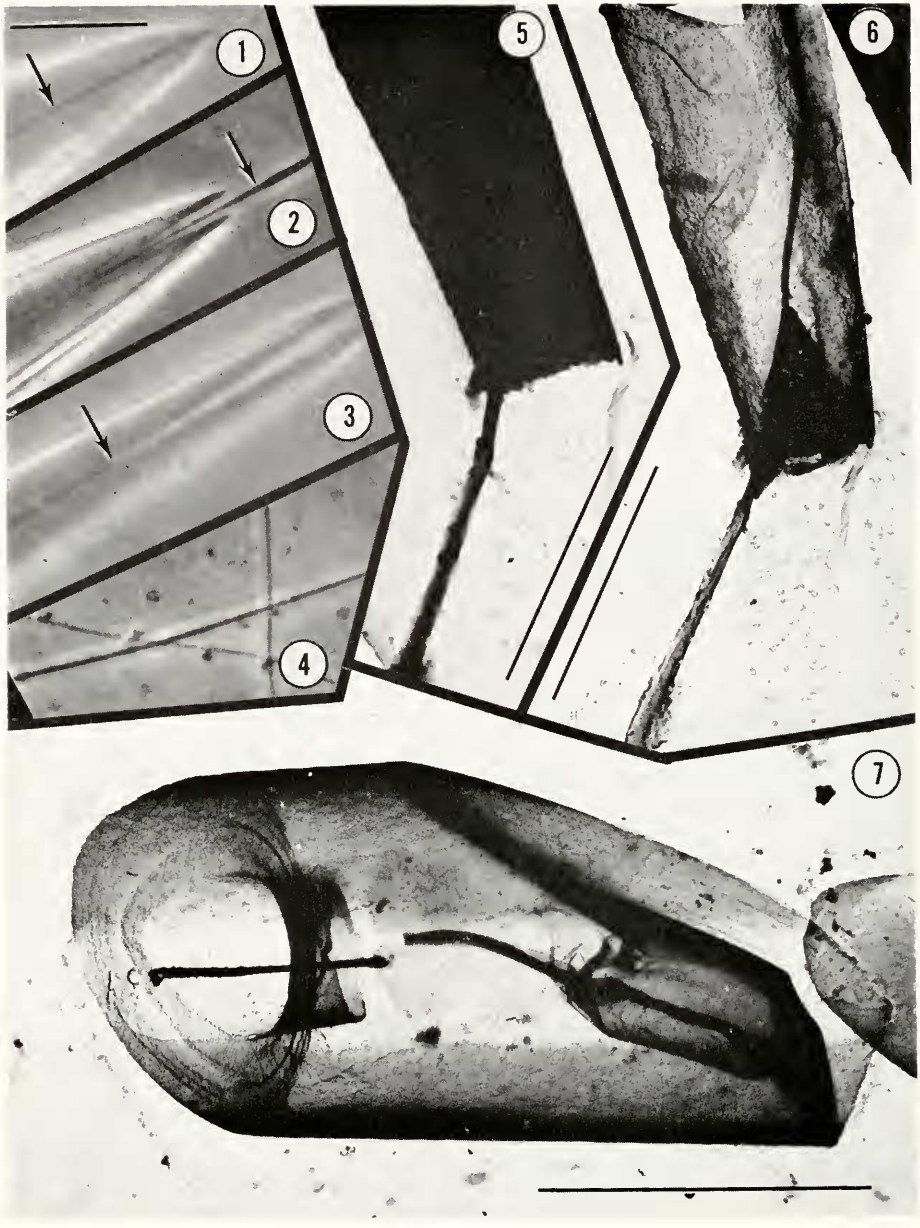
often displaced or obscured. The technique was modified in our laboratory to facilitate study of the filament. The spicule was partially removed by a brief etch in HF, exposing a portion of the axial filament. After rotary shadowing, the remaining spicule was removed by a second digestion in HF. The axial filament was held attached to the replica. Cleaned spicules were broken with the edge of a small spatula and sprinkled loosely onto a collodion-coated 200 mesh electron

TABLE I

Chemical analysis of spicules cleaned in boiling HNO_3

Component	Weight as % of spicule	Weight as % of residue	Atomic ratio to Si in residue (×100)
Part A: By loss of weight in 1 *N* HF at 55°			
$SiO_2 + H_2O$	95.7		
Residue	4.3	100.0	
Part B: By atomic absorption, expressed as the element determined			
Si	—	17.5	100.0
Na	0.65	13.7	94.0
K	0.124	2.57	10.5
Al	0.154	3.07	18.2
Ca	0.014	0.29	1.16
Fe	0.007	0.14	0.4
Part C: By combustion and gas chromatography of the oxide, expressed as the element determined			
N	0.073–0.57	1.46–11.5	
H	0.022–0.08	0.43–1.6	
C	0.016–0.042	0.32–0.83	
Part D: By semi-quantitative emission spectrography, expressed as the oxide of the element named			
B		0.6–6.0	
Ba		0.03–0.3	
Mg, Ni, Ti, Zr (each)		0.02–0.2	
Pb		0.01–0.1	
Sr		0.006–0.06	

microscope grid. One drop of aqueous 5% HF was placed on the gird. After 2-½ to 3 minutes the drop was removed by touching the edge of the grid with filter paper. In a similar manner the grid was washed in three changes of distilled water and dried at room temperature. A carbon film was evaporated *in vacuo* onto the specimen during two to three complete revolutions of the sample. The remaining spicule was removed by floating the grid, spicule side down, on a drop of HF for 15 minutes. The grid was again washed three times in distilled water in the same manner.



FIGURES 1–7

FIGURES 1–4. Phase contrast photomicrographs of spicules and axial filaments in HF during digestion, all same magnification, scale line = 20 μ.

FIGURE 1. Intact spicule at first contact with HF showing axial filament (arrow).

FIGURE 2. Digestion in 1 N HF alone leaves the axial filament (arrow) protruding from partially digested siliceous cylinder.

A technique was also devised for studying the ultrastructure of spicules in cross section. In general terms, lightly etched spicule sections were replicated with cellulose acetate. The replicas were removed and shadowed, and this negative image examined. Cleaned spicules were embeddd in Epon 812 and polymerized at 60° for 48 hours. The blocks were cut with a diamond saw so as to expose cross sections of a maximum number of spicules. The sawed surface was polished with Barnesite (Edmund Scientific Company) polishing compound and cleaned in an ultrasonic cleaner with distilled water. A negative replica of the polished surface was made by evaporating a drop of 8% cellulose acetate in acetone on the surface. After about 4–6 hours, the dry plastic negative replica was stripped off. The replica was shadowed with Pt-C ($\tan^{-1} \frac{1}{3}$) and carbon coated. Spicule-containing areas were cut out, placed on 200 mesh electron microscope grids, and the plastic dissolved with acetone in a vapor condensation washer. The embedded spicules were then etched for 30 seconds in 5% HF and replicated again as before. Multiple replicas were made of the same surface. Specimens were studied in a Siemens Elmiskop I at 80 KV accelerating voltage.

Observations

Chemical analysis

The results of the three types of chemical analyses are all summarized in Table I. The first column shows the composition for the whole spicules, the second for the non-volatile residue after 1 *N* HF digestion. The table compares the amounts of the various major elemental components on the basis of weight as a percentage of total spicule (column 1), weight as a percentage of the non-volatile residue after HF (column 2), and atoms per atom of silicon in the residue (column 3). The spicules would appear to be primarily silicic acid (or silica, since the H_2O content was not directly determined). The residue may contain alkali silicates, but to have all the sodium and potassium present as silicate would require more silicon than is found. Were there very little alkali silicates in the residue there would be sufficient silicon for all the other cations to be present as metal silicates. The proportion C:H:N in the residue is between 1:50:6 and 1:100:260 as calculated from the range of values from the two portions of the inhomogeneous residue. In both cases the total carbon is in the range of 1% of the residue; therefore, less than 2% of the residue could be organic matter of the 40% C composition expected of proteins and carbohydrates. Since the N:H ratio is so high it seems unlikely that the N is present as an amine, but more probably as a nitrate.

Figure 3. Digestion in 1 *N* HF in the presence of citric acid removes the axial filament. The axial filament may be preferentially removed deep into the siliceous cylinder; arrow marks retreating tip of axial filament. Compare to Figure 1.

Figure 4. After completed digestion in HF, axial filaments remain (3 shown).

Figure 5. Electron micrograph of a spicule after partial removal in HF showing an axial filament protruding from the end, scale line = 10 μ.

Figure 6. Same spicule as in Figure 5 after rotary replication and complete removal in HF. The axial filament can be seen extending from the tip of the conical etch pit, scale line = 10 μ.

Figure 7. Spicule fragment partially removed in HF, rotary shadowed, and then completely removed in HF. The etch pit is only partially replicated but the axial filament is evident, scale line = 10 μ.

Light microscopy

Measurements with an eyepiece micrometer agree with the published dimensions for the styles of *Acarnus erithacus,* namely 20μ × 340μ.

An axial structure can be seen inside intact spicules at high magnification with phase optics. Figure 1 is a photomicrograph showing such a structure. It does not appear to be smooth but angular in profile. It does appear to be cylindrically symmetrical. During digestion in 1 *N* or 2 *N* HF, the siliceous outer portion of the spicule is removed and the axial filament remains (Figs. 2 and 4). The axial filament may be observed to kink, wave in the fluid, or lie on the substratum. Prolonged exposure to 2 *N* HF does not visibly alter the filament.

By contrast to the persistence of the filament in HF alone, when citric acid is added to the HF (from 2 to 4.8 *M* citric) both the outer and axial portions of the spicule vanish within 15 minutes. The axial filament may even be destroyed prior to the siliceous cylinder. This is illustrated by a comparison of Figure 2 (HF alone) and Figure 3 (HF and citric). The siliceous outer portion of the spicules is at about the same stage of digestion.

Electron microscopy of whole spicules

With the rotary shadow technique it was possible to study the axial filament from a spicule that had been partially removed, and then to remove the HF-sensitive material completely and study the same region again by examining the carbon replica made during the rotary shadowing. Figure 5 shows a partially removed spicule with a filament extending from the end. The eccentric position of the filament will be understood after comparison with Figure 6, which is a replica of the *same spicule* seen in Figure 5 after the spicule was completely removed. The internal relationship of the filament and spicule is revealed. The removal of siliceous material by HF occurs most rapidly in the interior of the spicule, producing a conical etch pit. The filament can be seen to lie along the face of this cone extending outside the spicule from the edge of the cone's base and extending inside the spicule from the cone's apex. The carbon of the replica apparently is attached to the filament holding it in position as it was prior to removal of the second portion of spicule. For deep etch pits at unfavorable orientations to the carbon during replica formation, or for thin carbon coats, the filament may not be attached to the replica on the surface of the spicule. As Figure 7 shows, the interior portion of the filament is displaced slightly in such circumstances.

Electron microscopy of spicule cross sections

These figures are derived by shadowing a *negative replica* of the surface of the spicules so that protrusions on the spicule become pits on the replica, and pits on the spicule are protrusions on the replica. The conical etch pit already seen in the whole mount (positive shadow replica) is here a conical protrusion with a long shadow free of platinum. The etching time for the embedded spicules was necessarily shorter than for the partial removal of whole spicules. The result is much smaller etch pits and finer detail than was seen with the whole-spicule preparations.

Figure 8 is a cross section of a style, the major macrosclere of *Acarnus crithacus* that was polished but not etched. Similar preparations after light etching with HF are shown in Figures 9 and 10. Occasionally, the axial filament

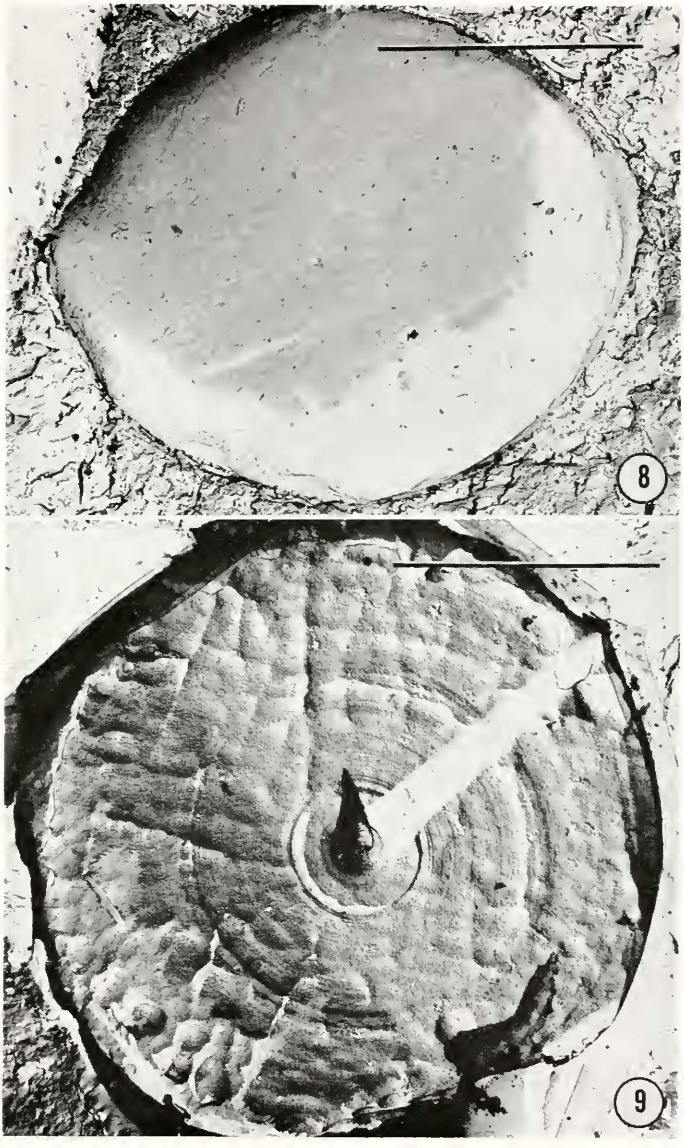


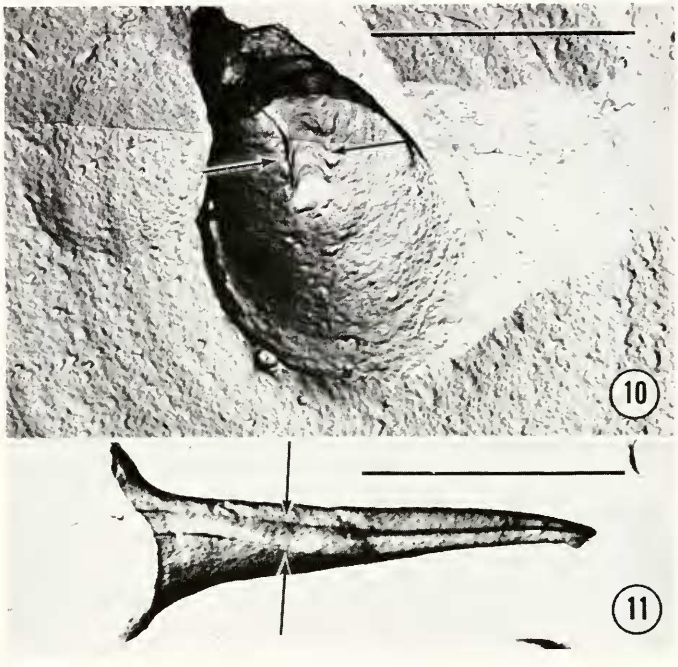
FIG. 8–9

FIGURE 8. Electron micrograph of a spicule cross section after polishing but no etch; C/Pt shadowed negative replica, scale line = 10 μ.

FIGURE 9. Spicule cross section after polishing and a 30-second etch in 5% HF. The center etches very rapidly and forms a conical etch pit. Closely spaced concentric rings are also evident; C/Pt shadowed negative replica, scale line = 10 μ.

may be seen as a detail in the etch-pit cone (Fig. 10). Oblique sections through spicules provided supplementary evidence of the axial position of this filament, as shown in Figure 11. The shape of the axial filament is not clear from these observations. It may be either triangular or circular in section. The filament appears to be about 0.4–0.6 μ in diameter.

The negative replicas exhibit circular surface protrusions (Figs. 9 and 10), indicating the existence of indentations produced by etching on the surface of the spicule. The more pronounced, that is, deeper, rings may correspond to those



FIGURES 10–11

FIGURE 10. Replica of an axial filament in an etch pit (arrows); C/Pt shadowed negative replica, scale line = 1 μ.

FIGURE 11. Replica of an etch pit in oblique section showing the axial filament (arrows); C/Pt shadowed negative replica, scale line = 1 μ.

described by Bütschli (1901) from light microscopy of stained fractured spicules. Several shallower grooves occur between and concentric with the deeper clefts. The rings are usually more pronounced closer to the center of the spicule. This is correlated with the more rapid removal of material in this region during etching that is expressed as the conical etch pit already mentioned above. The rings are somewhat irregularly spaced. The thinnest ones are about 0.2–0.3 μ thick.

DISCUSSION

The pattern of chemical composition revealed by our analysis is in general agreement with the literature. The bulk of the siliceous spicule is cation-free

oxide of silicon, presumably $(SiO_2)_xH_2O$. The present data provide no value for the ratio of SiO_2 to water. Values have been reported in the literature ranging from 3 to 5. (Minchin reviews the literature before 1909; see also Jørgensen, 1944).

There is little agreement in the literature on the cation composition of siliceous spicules, presumably reflecting real differences among the forms examined (Minchin, 1909). To compare two marine Demospongiae, the present values are in agreement with Bütschli's for sodium and the traces of aluminum, though he found much more magnesium.

From the composition and the density there is no reason to question the use of the term "spicopal" to refer to the siliceous portion of the spicule (Vosmaer and Wijsman, 1905), that is, to presume an amorphous silicic acid with traces of various cations with properties similar to the mineral opal.

As to the nature of the axial filament, there can be little question now that the siliceous macroscleres have a component part that is both axial and HF-resistant. The filament can be directly observed before and during etching. The high magnification light microscopy leads us to conclude that the filament visible by phase microscopy is not derived from the outer surface of the spicule by collapse of a net or sheath. Nor can we see how it could be derived by the coalescence of isolated particles within the siliceous matrix that come together during dissolution of the spicule. Organic matter on the native spicule surface would presumably have been destroyed by the 4-hour digestion in boiling concentrated nitric acid. The direct observation during solution of the spicule in HF reveals an object protruding from the end of the retreating spicule that has the same dimensions as a line found within the spicule prior to etch.

What portion of the axial filament may be protein or other organic material? The destruction of axial filament in mixed citric-hydrofluoric acid indicates only that a chelatable cation contributes to the stability of the filament. Other similar organic substances have been characterized; in particular a presumably unrelated factor involved in cell aggregation has been purified from sponge (Moscona, 1968). Its insolubility depends on the presence of Ca^{++} or other divalent cations. The most direct evidence of the possibility of a primarily organic filament comes from the CHN analysis. The carbon present (between 0.32 and 0.83% of the residue) would be sufficient to account for at least 0.8% of the residue as organic matter with 40% carbon, the range found in carbohydrates and proteins. As a fraction of the total spicule weight, this would be at least 4.0×10^{-4}. The filament visible in electron micrographs appears to be about 0.5 μ in diameter and the spicules are about 20 μ in diameter. The ratio of filament cross section to spicule cross section, that is, the ratio of radii squared, has a value of 6.3×10^{-4}. Mass density of the organic material would be expected to be around 1.27 (gelatin) to 1.53 (starch) and of the spicopal about 1.96 (whole spicules) to 2.3 (opal). Thus a given mass of organic material would fill about 1.5 times the volume of the same mass of opal.

When the carbon found (expressed as the organic fraction of the total spicule), 4.0×10^{-4}, is multiplied by this mass density correlation factor, 1.5, the product is 6.0×10^{-4}. That is, organic matter makes up by weight 0.4 parts per thousand of the whole spicule, and that much is expected to occupy 0.6 parts per thousand

by volume. We observe a structure that occupies about 0.63 parts per thousand by volume. We are led to conclude that there is sufficient carbon detected to provide a major fraction of the visible axial filament as organic matter with the carbon content of protein or carbohydrate. It is important to emphasize that this must be a minimum estimate since the harsh cleaning procedure, boiling concentrated nitric acid, while removing the organic material from the native surface of the spicule might penetrate into the open tip of some spicules and remove some material.

The data of Travis, François, Bonar, and Glimcher (1967) deserve comment at this point. As part of a broad comparative study of the organic material associated with the mineralized skeletons in animals, they examined the amino acid composition of an HF residue of siliceous spicules. They cleaned the spicules with distilled water only. Such a treatment is not likely to remove the collagen fibers that bind spicules in place, to say nothing of less specific cellular debris. Therefore, their data apply to a mixture of surface and axial material. It may well be that the surface material is more important in controlling the actual deposition of the siliceous body of the spicule.

A second recent paper of importance is by Drum (1968). His conclusions on organic material rest primarily on incineration data. Unfortunately, organic material is not the only material volatilized by such a procedure.

All three microscopy techniques used in this study indicate the existence of an HF-resistant filament that is axial in position. From the chemical analysis there would seem to be sufficient organic matter to account for the filament as carbohydrate or protein. Organic axial filaments have been demonstrated in calcareous spicules (Minchin and Reid, 1908). Jones (1967) refutes much of that work, contending that the filaments are preparation artifacts and at best are impure calcite in the intact spicule. The present work does not appear to be subject to that kind of criticism.

Concentric rings are revealed by gentle etching of spicule cross sections. From fracture studies the earlier workers, reviewed by Minchin (1909), had concluded that the spicule was composed of concentric lamellae. Schulze (1904) observed a similar ring pattern in Hexactinellids which he interpreted as lamellae of organic matter alternating with siliceous material.

According to Bütschli, the concentric strata were layers of different refractility. The lowered refractility, he surmised, arose from a minutely alveolar structure. Our figures show that some of the rings are clefts and some are dikes, thus differing in etch sensitivity. It is not clear how such a pattern of concentric but irregularly spaced zones of etch-resistant and etch-sensitive material could be related to Schulze's pattern of regular rings, presumably of organic material. Bütschli's notion of differences in compactness appears to be clearly related to the kind of pattern we see. However, if microalveoli are present, they must be below the resolution of our replica technique.

We must further emphasize the discrepancy in scale of the parts of the 20-μ diameter spicule as reported here for *Acarnus* and as reported by Bütschli for *Tethya* and *Geodea*. His axial filament is about 2.4 μ in diameter, that is, a fraction 0.12 of spicule diameter. His concentric rings are about 1.8 μ thick, that is, 11 rings for the entire 20-μ diameter spicule. Our filament diameter is

about one-fourth of his, both in absolute terms and as a fraction of spicule diameter. Our rings are similarly smaller than his by a factor of 0.14. Rings of this small size are at the limit of resolution of the light microscope. It is easily understandable that Bütschli would not have detected these as individual rings.

We cannot rule out the attractive possibility that subtle differences in composition or physical structure of the spicopal give rise to both the differences in etch sensitivity that we report and the negative birefringence that others have reported (Minchin, 1909).

Investigations are underway in this laboratory to determine the spatial distribution of chemical elements within the spicule.

We gratefully acknowledge the expert assistance of several colleagues for providing chemical analyses; W. H. Grieve for gas chromatography, R. H. Hall for emission spectrography and T. J. Gomoll for atomic absorption.

Summary

Siliceous spicules from *Acarnus erithacus* were studied using electron and light microscopy in conjunction with chemical analyses by gas chromatography, emission spectrography, and atomic absorption. Chemical data correlated with microscopy measurements indicate that there is sufficient carbon to provide a major fraction of the axial filaments as organic matter (assuming 40% carbon). The concentric ring structure reported earlier from light microscopy was studied on the electron microscopic level by utilizing carbon-platinum replicas of HF etched spicule cross sections. Ring spacings as small as 0.2–0.3 μ were detected using this technique. Correlation of chemical composition and fine structure is discussed with respect to the axial thread and the siliceous portions of the spicule.

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